Ph.D. Synopsis

Elucidating the ameliorative role of Mimusops elengi extract concerning gut microbiota in a letrozole-induced rat model of polycystic ovarian syndrome.

Submitted by

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1. State of the art

Polycystic Ovarian Syndrome (PCOS) is one of the most complex neuroendocrine, metabolic, and reproductive illness which affects approximately 4% to 20% of women of reproductive age (Cooney and Dokras 2018). In 1935, Stein and Leventhal were the first to characterize polycystic ovarian syndrome in the form of a study titled "Amenorrhoea associated with polycystic ovaries." This discovery has since prompted an array of medical research (Stein 1935). Numerous sets of diagnostic criteria have been established for the confirmation of PCOS due to the complexity of this condition. These sets include the Androgen Excess-PCOS Society (AE-PCOS) (Azziz et al. 2009), the Rotterdam criteria (Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). 2004), and the National Institutes of Health (NIH) criteria (Zawadzki 1992). PCOS is defined as having two of the following three characteristics, after ruling out other aetiologies: i) polycystic ovaries; ii) hyperandrogenism; and iii) oligo-and/or anovulation (Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). 2004). PCOS is a metabolic illness with other systemic consequences such as obesity, hyperlipidaemia, fatty liver, type 2 diabetes, hypertension, anxiety, depression, obstructive sleep apnoea, endometrial cancer, and cardiovascular disease (Cooney and Dokras 2018; El Hayek et al. 2016). As a multisystem condition including genetic, epigenetic, and environmental components, the pathogenesis of PCOS is yet unknown (Bruni, Capozzi, and Lello 2022).

Recent advances in next-generation sequencing technology have shown a correlation between altered gut microbiome and ailments such as neurological disorders, autoimmune diseases, cardiovascular diseases, and metabolic diseases. Because they are active agents in preserving the integrity of the gut epithelium, producing vitamin B12, and forming short-chain fatty acids through several metabolic and neurological processes, they have consequently been given priority in the search for therapeutic drugs, especially in these diseases (Durack and Lynch 2019). Tremellen and Pearce were the first to put up a microbiological theory and clarify the possible contribution of gut bacteria to the development of PCOS (Tremellen and Pearce 2012). According to recent research, women with PCOS have altered gut microbiome composition, and these alterations are linked to the metabolic and clinical traits of PCOS (Guo et al. 2016; Liu et al. 2017). Gut bacteria and PCOS may be related through several different pathways. According to Wang et al., these bacteria affect the course of PCOS by modifying the synthesis of metabolites, gut-brain mediators, cytokines, and hormones (Wang et al. 2021). Poor diet-

induced gut dysbiosis raises mucosal permeability, which limits the growth of protective bacteria and facilitates the entry of lipopolysaccharides from Gram-negative colonic bacteria into the bloodstream (Tremellen and Pearce 2012). According to certain studies (Insenser and Escobar-Morreale 2013; Jobira et al. 2020; Qi et al. 2019), there was a decrease in Bacteroidaceae and Porphyromonadaceae in PCOS, but an abundance of Streptococcaceae, *Bacteroides vulgates*, *Parabacteroides*, and *Clostridium*. It is unclear, nonetheless, exactly where PCOS and gut microbiome are related. In mice models with letrozole-generated PCOS, Kelley *et al*. observed a profound differentiation and phylogenetic variation in the gut microbiome that was diet-independent, not observed in the placebo group. They found that therapy with letrozole was associated with alterations in the populations of bacteria belonging to the phyla Firmicutes and Bacteroidetes, which had been seen in various mouse models before. Moreover, they saw that mice's microbiome changed after receiving letrozole, which led to weight gain, suggesting that changes to the gut microbiome may cause an increase in adiposity (Kelley et al. 2016). According to Marx and Mehta clomiphene citrate, metformin, and tamoxifen are currently the most often used medications to treat PCOS (Marx and Mehta 2003). Plant-based medications, especially those containing oestrogen (phytoestrogens), are believed to be significantly more effective in treating PCOS as compared to pharmaceuticals due to their less harmful side effects (Beck, Rohr, and Jungbauer 2005; Khani et al. 2011). According to the World Health Organisation (WHO), two-thirds of the world's population is being treated for a range of illnesses with traditional medicines.

Several studies have demonstrated the potential benefits of specific plants on the hormonal profile, including insulin sensitivity and ovarian activity in PCOS-affected rats. These include *Tephrosia purpurea* (Jitendra and Pravin 2012), *Aleo barbadensis* (Maharjan, Nagar, and Nampoothiri 2010), *Wistthania somnifera*, *Allium fistulous* (Lee et al. 2018), *Tribulus terrestris* (Saiyed et al. 2016), and *Phyllanthus muellerianus* (Ndeingang et al. 2019).

Mimusops elengi is a well-known traditional medicinal plant that grows to a height of 12 to 15 meters. It is also known as the Indian medlar tree or the Bakul tree. It may be found in tropical woods in South Asia and India, as well as the Western and Eastern Ghats. Because *Mimusops elengi* contains several active phytoconstituents, it possesses a variety of biological and pharmacological activities (Gami, Pathak, and Parabia 2012; Kadam et al. 2012). It possesses antibacterial, antihemorrhoidal, antifungal, free radical scavenging, antihyperglycemic, antineoplastic, gastroprotective, diuretic, antiviral, and cytotoxic effects (Baliga et al. 2011; Gami, Pathak, and Parabia 2012; Hadaginhal et al. 2010; Kadam et al. 2012).

Despite having all of these properties, till now, *Mimusops elengi* has not been evaluated for the pathophysiology associated with PCOS in an animal model. *Mimusops elengi* possesses therapeutic properties in all of its parts including leaves, flowers, fruits, seeds, and barks. So, we begin our investigation with the flowers in this study. Using a rat model of PCOS induced by letrozole, we will investigate whether *Mimusops elengi* flower extract can help alleviate the different symptoms of PCOS.

2. Problems to be investigated

Concerning the rising frequency of PCOS and its related physical and mental issues, as well as the role of gut microbiota alterations in the development of this ailment, our study will evaluate the potential therapeutic benefits of *Mimusops elengi* flower extract in treating PCOS abnormalities in a rat model.

3. Objective

The present research can be subdivided into the following broad objectives:

3.1. Preparation of flower extract and its phytochemical studies.

The preliminary goal of this project is to prepare an aqueous extract of *Mimusops elengi* flower and analyse its phytochemical constituents by GC-MS.

3.2. Induction of Polycystic ovary in rats and evaluation of consequent oestrous stages.

The focus of this objective is to induce polycystic ovary in rats using letrozole. Letrozole, a nonsteroidal aromatase inhibitor which raises androgen levels. Letrozole also disrupts the oestrous cycle and elevates LH and testosterone (T) levels and ovulation in female rats.

3.3. The impact of *Mimusops elengi* flower extract on the gut microbiome of rats along with biochemical and histochemical parameters.

The focus of this objective is to assess the impact of *Mimusops elengi* on the composition of the gut microbiome in control and treated rat models to see their effect on the diversity of bacteria that influences PCOS symptoms. Further biochemical and histochemical parameters such as haematological, hormonal, lipid, interleukins, angiogenesis markers, and antioxidant profiles will be analysed.

4. Hypothesis

The research community currently feels that there is an urgent need for the therapeutic management of PCOS. It is commonly known that using pharmaceuticals for therapy in an allopathic manner is neither reliable nor economical. Thus, there is a requirement to discover an herbal remedy for this illness that resembles a threat. The present plant

(*Mimusops elengi*) contains several phytoconstituents that cure a variety of ailments. So, we hypothesized that *Mimusops elengi* extract may play a potent role in the management of PCOS.

5. Research Design and methodology

5.1. Objective 1 methodology: Preparation of flower extract and phytochemical studies.

Briefly, the following experiments are planned for this objective:

5.1a. Preparation of flower extract.

Study design: *Mimusops elengi* will be collected in Ranchi, Jharkhand, between April and June. Fresh, clean flowers will be dried at 40°C in a hot air oven after being collected. Next, the flowers' petals and sepals will be separated. Using an electric grinder, each component will be ground into a fine powder before being macerated in methanol for a week at room temperature. The solvent extracts will then be evaporated using a rotary evaporator and filtered using Whatman filter paper to produce methanolic extracts of the petals and sepals of *M. elengi*.

5.1b. Analysis of the extract using GC-MS.

GC-MS (Gas chromatography-mass spectrometry) will be used to characterize the phytochemical components of the *Mimusops elengi* flower extract. By comparing the test samples' mass spectral patterns, peak area, peak height, and retention time (min) to those of known molecules and compounds preserved in the NIST (National Institute of Standards and Technology Library), the phytochemical content of the samples will be ascertained.

5.2. Objective 2 methodology: Induction of Polycystic ovary in rats and evaluation of consequent oestrous stages.

5.2a. Experimental animals

Study design: Healthy young female Wistar rats, between the ages of 10 and 12 weeks, will be obtained before the experiment. They will be fed commercial pellets and given unlimited access to tap water.

Mimusops elengi flower extract will be tested on thirty-six female Wistar rats to see how it affects PCOS-related symptoms. Briefly, the following experiments are planned for this objective as shown in **Figure 1.**

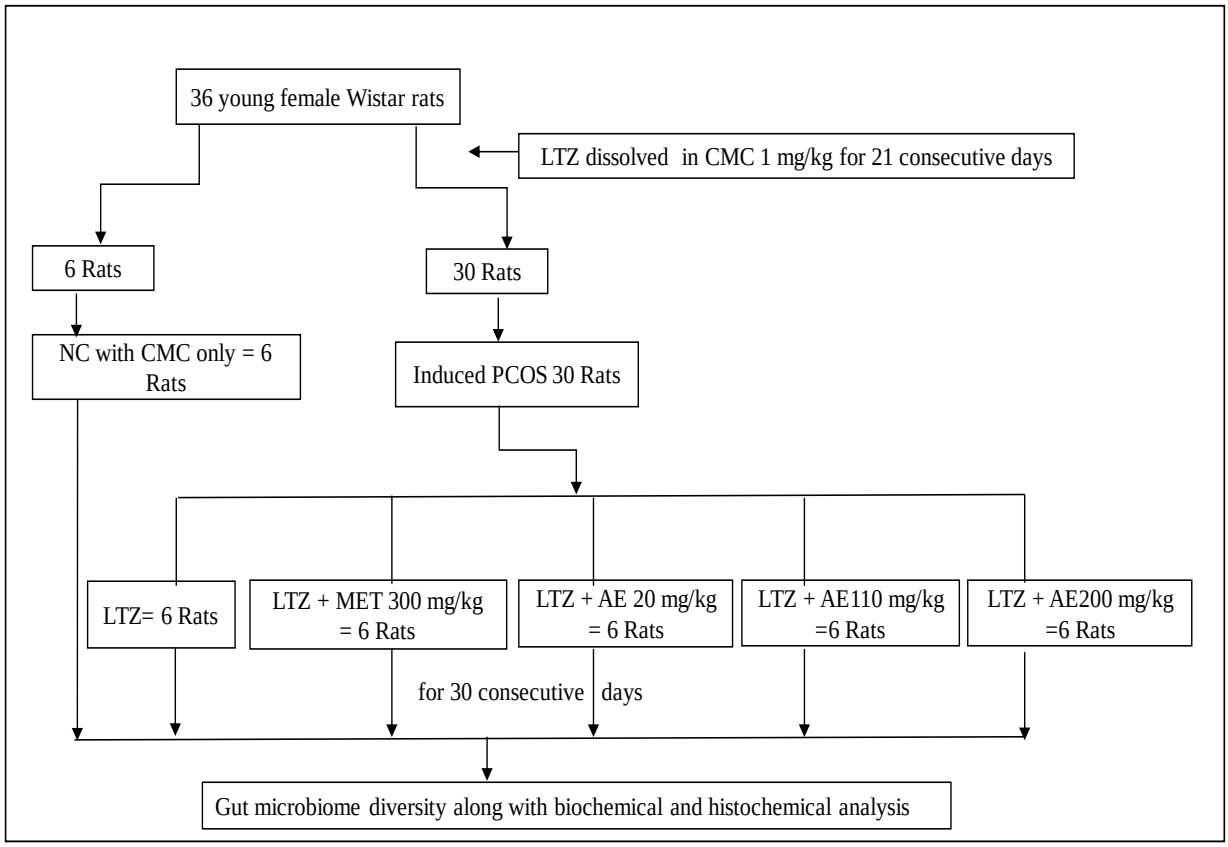
36 young female Wistar rats
LTZ dissolved in CMC 1 mg/kg for 21 consecutive days
6 Rats
30 Rats
NC with CMC only = 6 Rats
Induced PCOS 30 Rats
LTZ= 6 Rats
LTZ + MET 300 mg/kg = 6 Rats
LTZ + AE 20 mg/kg = 6 Rats
LTZ + AE110 mg/kg =6 Rats
LTZ + AE200 mg/kg =6 Rats
for 30 consecutive days
Gut microbiome diversity along with biochemical and histochemical analysis

Figure 1. Flow chart depicting work methodology. LTZ: Letrozole; CMC: Carboxy Methyl Cellulose NC: Normal Control; AE: Aqueous extract; MET: Metformin

Letrozole will be used to induce PCOS following the Ndeingang et al. approach (Ndeingang et al. 2019). It is a reversible inhibitor of aromatase; thus, its administration won't be stopped during this course of treatment. Therefore, letrozole will be given in addition to the extract of *Mimusops elengi* flowers during the treatment period to ensure PCOS induction in experimental animals. This will allow us to ascertain whether the extract alleviates PCOS symptoms even when the inducer is present.

5.2b. Determination of oestrus cycle

Every rat's vaginal smear will be microscopically inspected from the start of the treatments until the finish (Ndeingang et al. 2019). The kinds of cells seen in rat vaginal smears will be used to identify the stages of the oestrous cycle.

5.3. Objective 3 methodology: The impact of *Mimusops elengi* flower extract on the gut microbiome of rats along with biochemical and histochemical parameters.

Briefly, the following experiments are planned for this objective:

5.3a. Anthropometrical parameters

Study design: Throughout the investigation, weekly body weight changes will be monitored in the control, letrozole-induced PCOS, Metformin-treated, and *Mimusops elengi* flower extract-treated groups. The day of dissection will be used to assess the following: body length and weight increase, body mass index (BMI), the ratio of the abdominal circumference to the thoracic circumference (AC/TC), and the particular rate of body mass gain. Using standard measurement techniques, the diameter, weight, and organ index of the ovaries will also be assessed.

5.3b. Gut microbiome analysis

Study design: Samples of faeces from both control and treated rats will be collected, and complete DNA extracted from the samples will be sequenced using Next Generation sequencing technology to determine the microbiome compositions.

Faecal DNA extraction: To study the gut microbiome diversity, the metagenomic DNA will be isolated using Commercial Kit (Qiagen). DNA purity and quantity will be tested using Nano drop machine. The samples will be submitted to a commercial company for 16sRNA sequencing for analysing microbiomes present in faecal samples.

Analysis of metagenomics data

Bioinformatics data will be analyzed using commercial software (Qlucore Omics Explorer, https://qlucore.com). Qlucore Omics Explorer has been used by many scientists worldwide in more than 1000 publications (https://qlucore.com/publications).

5.3c Biochemical Analysis

Study design: An electronic glucometer will be used to measure blood glucose levels throughout the current investigation. The ELISA technique will be used to measure the levels of oestradiol, follicular stimulation hormone (FSH), and serum luteinizing hormone (LH). Additionally, commercially available kits will be used to analyse total cholesterol (TC), high-density lipoprotein triglycerides (TG), creatinine, urea, blood urea nitrogen (BUN), total protein, and C-Reactive protein CRP.

5.3d Histochemical analysis

Study design: After histological processing, prepared slides will be observed under a light microscope. Every ovarian follicle will be assessed based on its morphology and granulosa. We will also measure the thickness of the granulosa layer and peripheral theca, as well as the existence or absence of the corpus luteum.

Oxidative and anti-oxidant profile estimation

The ovarian tissue of both control and treated animals will be examined for the presence of antioxidant enzymes, specifically superoxide dismutase, malondialdehyde, reduced glutathione, and nitric oxide.

Interleukins analysis (Pro-inflammatory/inflammatory biomarkers)

The plasma IL-6 and TNF-α concentrations will be measured using ELISA, with commercial kits.

RT-PCR to detect expression levels of angiogenesis markers.

It has been demonstrated that ovarian angiogenesis plays a role in the development of PCOS pathogenesis. Real-Time quantitative PCR will be used to evaluate the expression of genes linked to angiogenesis indicators, including platelet-derived growth factor (PDGF)-A, vascular endothelial growth factor (VEGF), and angiopoietin in control and treated ovarian tissue samples.

5.4.Statistical analysis

The data will be analysed and reported as Mean ± Standard Error of Mean (SEM) using the GraphPad Prism. Statistical significance and group differences will be determined using one-way ANOVA, followed by the Tukey test for multiple comparisons. Differences will be considered significant at $p < 0.05$.

6. Tools To Be Used

1) Microscope
2) Autoclave
3) UV – vis Spectrometer
4) Biosafety Cabinet
5) Gas-chromatography
6) Glassware and plastic apparatus
7) Centrifuge
8) PCR
9) RT-PCR
10) Agarose gel electrophoresis
11) Gel-Doc system
12) SDS-PAGE electrophoresis

13) BOD and CO_2 Incubator

14) Electric grinder and Rotary Evaporator

7. Signification of study

Several treatments are currently being used to treat PCOS and trigger ovulation. However, there have been reports of serious side effects from these treatments. As a result, patients are turning more and more to herbal therapy that has few or no side effects. Various plants have been reported to possess antioxidant properties that effectively cure PCOS by raising sex hormone levels in the blood and lowering oxidative stress. Based on the available data, many phytoestrogen and antioxidant chemicals present in medicinal plants have the potential to alleviate symptoms of PCOS, making them useful treatments for this condition. It has been shown that the flowers and leaves of *Mimusops elengi* have phytoestrogen and antioxidants which might be effective in treating PCOS. This will be the first study to see the therapeutic benefits of *Mimusops elengi* extract in PCOS possibly by increasing microbiota diversity in the *M. elengi-treated* rat model.

8. Future aspect

In the future, we will study the molecular and *in-vivo* study by identifying key genes and their role in PCOS symptoms. Further, gut microbiota diversity will be explored by metagenomics and metabolic studies for the prevention, and treatment of PCOS.

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